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Remarks:
The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) A kanamycin resistance gene derived from microorganisms of the genus rhodococcus

(57) The present invention relates to a DNA derived from microorganisms of the genus Rhodococcus and conferring kanamycin resistance on hosts with a DNA sequence coding for the amino acid sequence of Sequence No. 1 or a polypeptide containing a partial sequence thereof. The kanamycin resistance gene of the present invention is useful to construct vectors for microorganisms of the genus Rhodococcus, particularly vectors for self-cloning of Rhodococcus rhodochrous.

EP 0 704 530 A2

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Description

The present invention relates to a gene derived from microorganisms of the genus Rhodococcus and conferring kanamycin resistance on bacteria, as well as a plasmid vector containing the same.

Microorganisms belonging to the genus Rhodococcus are known as bacterial catalysts that hydrate or hydrolyze nitriles to the corresponding amides or acids (Japanese Patent Publication No. 4873/92 and Japanese Laid-Open Patent Publication Nos. 91189/87, 470/90 and 84198/90), and in particular, microorganisms belonging to the species Rhodococcus rhodochrous possess nitrile-hydrating activity of extremely high performance (Japanese Laid-Open Patent Publication No. 470/90).

Under such circumstances, one of the present inventors found cryptic plasmids in a certain strain of the species Rhodococcus rhodochrous and constructed hybrid plasmid vectors to develop a host-vector system of the genus Rhodococcus (Japanese Laid-Open Patent Publication Nos. 148685/92, 64589/93 and 68566/93).

For construction of a self-cloning system of higher safety, it is also necessary to develop marker genes derived from microorganisms of the genus Rhodococcus. However, only arsenious acid and cadmium resistance genes derived from microorganisms of the species Rhodococcus rhodochrous are known as such drug resistance genes (Plasmid 23, 242-247 (1990)).

With the aim of establishing a self-cloning system of the genus Rhodococcus, the present inventors extensively studied drug resistance genes derived from microorganisms of the genus Rhodococcus, in particular the species Rhodococcus rhodochrous, so that they found the kanamycin resistance gene of the present invention.

That is, the present invention relates to a gene derived from microorganisms of the genus Rhodococcus and conferring kanamycin resistance on hosts, wherein said gene codes for the amino acid sequence of Sequence No. 1 or a polypeptide containing a partial sequence thereof.

The present invention furthermore relates to a gene conferring kanamycin resistance on a host comprising the DNA sequence of Sequence No. 2 or a DNA sequence which

(a) differs from said DNA sequence due to the degeneracy of the genetic code;
(b) hybridizes with said DNA sequence or the DNA sequence of (a); or
(c) represents a fragment, allelic or other variation of the above DNA sequence, whether said variation results in changes in the polypeptide sequence or not.

In this context, the term "hybridization" refers to conventional hybridization conditions, preferably to stringent hybridization conditions.

FIG. 1 shows a restriction enzyme map of plasmid pKM001.

FIG. 2 shows the construction of plasmid pKM002, pKM003 and pKM004.

FIG. 3 shows a restriction enzyme map of plasmid pKM011.

As the DNA donor in the present invention, mention may be made of kanamycin mutant KM-02 (deposited as FERM BP-5137 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan) which was obtained by spontaneous mutation of Rhodococcus rhodochrous ATCC 12674.

As the vectors used in cloning in the present invention, mention may be made of plasmid vectors including but not limited to E. coli vectors such as pTrc99A, pUC18, etc. and phage vectors such as λ gt11 etc. The host microorganisms include but are not limited to E. coli JM109, E. coli JM105, and Rhodococcus rhodochrous ATCC 12674.

Plasmids that provide plasmid vectors constructed of the kanamycin resistance gene of the invention with a region capable of replicating in microorganisms of the genus Rhodococcus include, but are not limited to, plasmids pRC001, pRC002, pRC003 and pRC004. The plasmids pRC001, pRC002, pRC003 and pRC004 are derived from Rhodococcus rhodochrous ATCC 4276, ATCC 14349, ATCC 14348 and IFO 3338, respectively, and these plasmids are described in the aforementioned Japanese Laid-Open Patent Publication Nos. 148685/92, 64589/93 and 68566/93, respectively.

The present kanamycin resistance gene derived from microorganisms of the genus Rhodococcus is useful to construct vectors for microorganisms of the genus Rhodococcus, particularly vectors for self-cloning of Rhodococcus rhodochrous.

The present invention is described in more detail with reference to the following examples, which however are not intended to limit the scope of the present invention.

Example 1

Cloning of Kanamycin Resistance Gene from Mutant KM-02 into E. coli JM109

(1) Preparation of genomic DNA from KM-02 and preparation of a DNA library

The KM-02 strain was cultured under shaking at 30 °C in 100 ml MY medium (0.5 % polypeptone, 0.3 % Bacto-yeast extract, 0.3 % Bacto-malt extract) and genomic DNA was prepared from the bacteria according to the method by Saito and Miura (Biochim. Biophys. Acta 72, 619 (1963)). A part of the resulting DNA was partially digested with restriction enzyme Sau3AI and then inserted into a BamHI site of E. coli vector pTrc99A to give a recombinant DNA library.

(2) Preparation of transformants and selection of recombinant DNA

The recombinant library prepared in step (1) was used to transform E. coli JM109 by the calcium chloride method, and transformants with resistance to kanamycin were selected in the following manner.

The transformants obtained above were plated onto LB agar medium (1 % Bacto-trypton, 0.5 % Bacto-yeast extract, 0.5 96 NaCl, 1.5 % agar) containing 40 μ g/ml kanamycin hydrochloride and 1 mM IPTG (isopropyl-β -thiogalactoside) and incubated overnight at 37 °C. The colonies occurring thereon were removed and applied onto the same agar medium, and their growth was confirmed.

A plasmid DNA was prepared from the thus obtained transformant according to the method by Birnboim and Doly (Nucleic Acid Res. 7, 1513-1523 (1979)) and designated pKM001. This plasmid was reintroduced into E. coli, and the resultant transformant with kanamycin resistance was designated JM109/pKM001 and deposited as FERM BP-5138 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology. IPTG was required for expression of Kanamycin resistance of E. coli JM109/pKM001.

(3) A restriction enzyme map of pKM001 and location of the kanamycin resistance gene

A restriction enzyme map of plasmid pKM001 obtained in step (2) was prepared (FIG. 1). Thereafter, this plasmid pKM001 was used for preparing plasmids of a smaller DNA fragment. The target gene-containing region was identified by the presence or absence of the kanamycin resistance of transformants prepared in the same manner as in step (2). During this process, plasmid pKM002 (FIG. 2) was constructed.

(4) Nucleotide sequencing

The nucleotide sequence of the kanamycin resistance gene in plasmid pKM002 was determined by Fluorescence Sequencer ALF II produced by Pharmacia (Sequence No. 3).

Example 2

Preparation of Hybrid (E. Coli-Rhodococcus) Plasmid Vector Carrying the Kanamycin Resistance Gene Derived from Rhodococcus Rhodochrous

A hybrid plasmid vector pK4, previously constructed by one of the present inventors by ligating Rhodococcus-derived plasmid pRC004 with E. coli vector pHSG299 and deposited as FERM BP-3731 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (Japanese Laid-Open Patent Publication Nos. 64589/93 and 68566/93), was used for preparing a 3.1 kb HindIII fragment containing the whole of pRC004 and a part of pHSG299, and the resulting fragment was ligated with the plasmid pKM002.

As a result, two plasmids carrying the insert in the opposite direction were obtained and designated pKM003 and pKM004, respectively (FIG. 2). These plasmids replicate in both the genus Rhodococcus and E. coli. Rhodococcus rhodochrous ATCC 12674 was transformed with these plasmids by electroporation, whereby a transformant capable of growing in MY medium containing 75μ g/ml kanamycin was obtained. The plasmids obtained from the transformant were the same plasmids as those introduced. Where microorganisms of the genus Rhodococcus were used as the host, the presence of IPTG was not required for expression of kanamycin resistance.

Example 3

Construction of Vector for Microorganisms of the Genus Rhodococcus

The hybrid plasmid vector pKM004 was cleaved with restriction enzyme KpnI to give a 4.3 kb KpnI fragment which was then self-ligated and introduced into Rhodococcus rhodochrous ATCC 12674 by electroporation. The resulting transformant showed the same degree of kanamycin resistance as did the transformant of Example 2. From this trans-

formant, a plasmid was obtained and designated pKM011 (FIG. 3).

SEQUENCE TABLE

Sequence No: 1

Length: 171

Sequence Type: amino acid

Topology: linear

Nature: protein

Origin

Microorganism: Rhodococcus rhodochrous

Strain: KM-02

Sequence:

Met Ser Asp Asn Gly Ser Gly Thr Thr Arg Pro Glu Gly Ala Pro Leu
1 5 10 15

Pro Arg Arg Ala Arg Ser Ser Arg Pro Ser Ala Gly Asn Ser Pro Ala
20 25 30

Pro Gly Arg Arg Ala Val Val Ala Lys Ser Arg Arg Arg Leu Ala Ala
35 40 45

Ala Pro Glu Ala Gly Thr Thr His Tyr Ser Ile Phe His Gly Asp Gln
50 55 60

Leu Ile Gly Phe Ile Gln Trp Tyr Glu Ala Glu Asp Asn Pro Asp Phe
65 70 75 80

Arg His Ala Gly Leu Asp Leu Phe Leu Asp Pro Asp Phe His Gly Arg
85 90 95

Gly Phe Gly Arg Glu Ser Ile Arg Val Leu Cys Ala His Leu Ile Asp
100 105 110

Asp Leu Ala Phe His Arg Leu Val Ile Asp Pro Glu Val Asp Asn Ser
115 120 125

Val Ala Ile Ala Cys Tyr Arg Ser Val Gly Phe Lys Asp Val Gly Val
130 135 140

Sequence No: 2

Length: 516

Nature: nucleic acid

Strand Form: double-stranded

Topology: linear

Origin

Microorganism: Rhodococcus rhodochrous

Strain: KM-02

Sequence:

ATG AGT GAC AAC GGC TCC GGA ACT ACG CGG CCC GAG GGT GCT CCT CTC	48
CCC CGT CGC GCC CGA TCA TCA CGC CCG TCT GCG GGC AAT TCA CCT GCA	96
CCC GGA CGT CGT GCA GTG GTG GCA AAA TCC CGA CGA CGA CTG GCT GCG	144
GCG CCA GAA GCC GGA ACC ACG CAC TAC AGC ATC TTC CAC GGC GAC CAA	192
CTG ATC GGC TTC ATC CAG TGG TAC GAA GCG GAA GAC AAC CCC GAC TTC	240
CGC CAC GCC GGG CTC GAC TTG TTC CTC GAT CCC GAC TTC CAC GGC CGA	288
GGG TTC GGT CGC GAA TCG ATT CGC GTG CTG TGT GCC CAC CTG ATC GAC	336
GAC CTC GCA TTC CAC CGT CTG GTC ATC GAC CCG GAG GTC GAC AAC TCC	384
GTC GCC ATC GCG TGC TAC CGA TCG GTG GGG TTC AAA GAC GTC GGG GTG	432
ATG CGC GAG TAT TCG CGA GAT CGC CAT GGT GTG TGG AAG GAC GGA CTG	480
CTG ATG GAT CTG CTC GCA CGG GAA TTC ATC CGC TGA	516

Sequence No: 3

Length: 748

Sequence Type: nucleic Acid

Strand Form: double-stranded

Topology: linear

Origin

Microorganism: Rhodococcus rhodochrous

Strain: KM-02

Sequence:

GGATCCGGGG TCGTCGCCCA CCAGGATGGT ACCCAAGCCG GGTGTGATGC CCTCTGCCTT 60

CGAGCGCTCA CCCGCACCTT CAGGTCTTCG AAGATTTCGT CGCGGGTAGC TTTGCCGTCG 120

AGGATCGTTG CAGTCACGGC GACCATTGTT CCAGGTTAGG GTCGATGAGT GACAACGGCT 180

CCGGAACTAC GCGGCCCGAG GGTGCTCCTC TCCCCCGTCG CGCCCGATCA TCACGCCCGT 240

CTGCGGGCAA TTCACCTGCA CCCGGACGTC GTGCAGTGGT GGCAAAATCC CGACGACGAC 300

TGGCTGCGGC GCCAGAAGCC GGAACCACGC ACTACAGCAT CTTCCACGGC GACCAACTGA 360

TCGGCTTCAT CCAGTGGTAC GAAGCGGAAG ACAACCCCGA CTTCCGCCAC GCCGGGCTCG 420

ACTTGTTCCT CGATCCCGAC TTCCACGGCC GAGGGTTCGG TCGCGAATCG ATTCGCGTGC 480

TGTGTGCCCA CCTGATCGAC GACCTCGCAT TCCACCGTCT GGTCATCGAC CCGGAGGTCG 540

ACAACTCCGT CGCCATCGCG TGCTACCGAT CGGTGGGGTT CAAAGACGTC GGGGTGATGC 600

GCGAGTATTC GCGAGATCGC CATGGTGTGT GGAAGGACGG ACTGCTGATG GATCTGCTCG 660

CACGGGAATT CATCCGCTGA TCGACTGGGA CGAGTTCGAA AGGACCGACA TCATGTTGCT 720

GGACAAGGAA TTCACGGCCA CCCTGCAG 748

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Nitto Chemical Industry Co., Ltd.
(B) STREET: 5-1, Marunouchi 1-chome, Chiyoda-ku
(C) CITY: Tokyo
(E) COUNTRY: Japan
(F) POSTAL CODE (ZIP): 100

(ii) TITLE OF INVENTION: A kanamycin resistance gene derived from microorganisms of the genus rhodococcus

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: EP 95 11 2298.5
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: JP 201582/1994
(B) FILING DATE: 04-AUG-1994

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 171 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rhodococcus rhodochrous
(B) STRAIN: KM-02

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Met Ser Asp Asn Gly Ser Gly Thr Thr Arg Pro Glu Gly Ala Pro Leu
1               5                  10                  15

Pro Arg Arg Ala Arg Ser Ser Arg Pro Ser Ala Gly Asn Ser Pro Ala
            20                  25                  30

Pro Gly Arg Arg Ala Val Val Ala Lys Ser Arg Arg Arg Leu Ala Ala
```

```
                35                  40                  45

Ala Pro Glu Ala Gly Thr Thr His Tyr Ser Ile Phe His Gly Asp Gln
    50                  55                  60

Leu Ile Gly Phe Ile Gln Trp Tyr Glu Ala Glu Asp Asn Pro Asp Phe
65                  70                  75                  80

Arg His Ala Gly Leu Asp Leu Phe Leu Asp Pro Asp Phe His Gly Arg
                85                  90                  95

Gly Phe Gly Arg Glu Ser Ile Arg Val Leu Cys Ala His Leu Ile Asp
            100                 105                 110

Asp Leu Ala Phe His Arg Leu Val Ile Asp Pro Glu Val Asp Asn Ser
        115                 120                 125

Val Ala Ile Ala Cys Tyr Arg Ser Val Gly Phe Lys Asp Val Gly Val
    130                 135                 140

Met Arg Glu Tyr Ser Arg Asp Arg His Gly Val Trp Lys Asp Gly Leu
145                 150                 155                 160

Leu Met Asp Leu Leu Ala Arg Glu Phe Ile Arg
                165                 170
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 516 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rhodococcus rhodochrous
(B) STRAIN: KM-02

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
ATGAGTGACA ACGGCTCCGG AACTACGCGG CCCGAGGGTG CTCCTCTCCC CCGTCGCGCC      60

CGATCATCAC GCCCGTCTGC GGGCAATTCA CCTGCACCCG GACGTCGTGC AGTGGTGGCA     120

AAATCCCGAC GACGACTGGC TGCGGCGCCA GAAGCCGGAA CCACGCACTA CAGCATCTTC     180

CACGGCGACC AACTGATCGG CTTCATCCAG TGGTACGAAG CGGAAGACAA CCCCGACTTC     240

CGCCACGCCG GGCTCGACTT GTTCCTCGAT CCCGACTTCC ACGGCCGAGG GTTCGGTCGC     300

GAATCGATTC GCGTGCTGTG TGCCCACCTG ATCGACGACC TCGCATTCCA CCGTCTGGTC     360
```

ATCGACCCGG AGGTCGACAA CTCCGTCGCC ATCGCGTGCT ACCGATCGGT GGGGTTCAAA 420

GACGTCGGGG TGATGCGCGA GTATTCGCGA GATCGCCATG GTGTGTGGAA GGACGGACTG 480

CTGATGGATC TGCTCGCACG GGAATTCATC CGCTGA 516

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 748 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rhodococcus rhodochrous
(B) STRAIN: KM-02

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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CGAGCGCTCA CCCGCACCTT CAGGTCTTCG AAGATTTCGT CGCGGGTAGC TTTGCCGTCG 120

AGGATCGTTG CAGTCACGGC GACCATTGTT CCAGGTTAGG GTCGATGAGT GACAACGGCT 180

CCGGAACTAC GCGGCCCGAG GGTGCTCCTC TCCCCCGTCG CGCCCGATCA TCACGCCCGT 240

CTGCGGGCAA TTCACCTGCA CCCGGACGTC GTGCAGTGGT GGCAAAATCC CGACGACGAC 300

TGGCTGCGGC GCCAGAAGCC GGAACCACGC ACTACAGCAT CTTCCACGGC GACCAACTGA 360

TCGGCTTCAT CCAGTGGTAC GAAGCGGAAG ACAACCCCGA CTTCCGCCAC GCCGGGCTCG 420

ACTTGTTCCT CGATCCCGAC TTCCACGGCC GAGGGTTCGG TCGCGAATCG ATTCGCGTGC 480

TGTGTGCCCA CCTGATCGAC GACCTCGCAT TCCACCGTCT GGTCATCGAC CCGGAGGTCG 540

ACAACTCCGT CGCCATCGCG TGCTACCGAT CGGTGGGGTT CAAAGACGTC GGGGTGATGC 600

GCGAGTATTC GCGAGATCGC CATGGTGTGT GGAAGGACGG ACTGCTGATG GATCTGCTCG 660

CACGGGAATT CATCCGCTGA TCGACTGGGA CGAGTTCGAA AGGACCGACA TCATGTTGCT 720

GGACAAGGAA TTCACGGCCA CCCTGCAG 748

Claims

1. A gene derived from a microorganism of the genus Rhodococcus and conferring kanamycin resistance on a host, said gene coding for the amino acid sequence of Sequence No. 1 or a polypeptide containing a partial sequence thereof.

2. The gene according to claim 1, comprising the DNA sequence of Sequence No. 2 or a partial sequence thereof.

3. A gene conferring kanamycin resistance on a host and comprising a DNA sequence which

   (a) differs from the DNA sequence of claim 2 in the codon sequence due to the degeneracy of the genetic code;
   (b) hybridizes with the DNA sequence of claim 2 or section (a), above; or
   (c) represents a fragment, allelic or other variation of the DNA sequence of claim 2, whether said variation results in changes in the polypeptide sequence or not.

4. The gene according to any one of claims 1 to 3, wherein the host microorganism is a microorganism of the genus Rhodococcus or Escherichia coli.

5. A plasmid vector comprising a gene according to any one of claims 1 to 4 and a DNA region capable of replicating in a microorganism of the genus Rhodococcus.

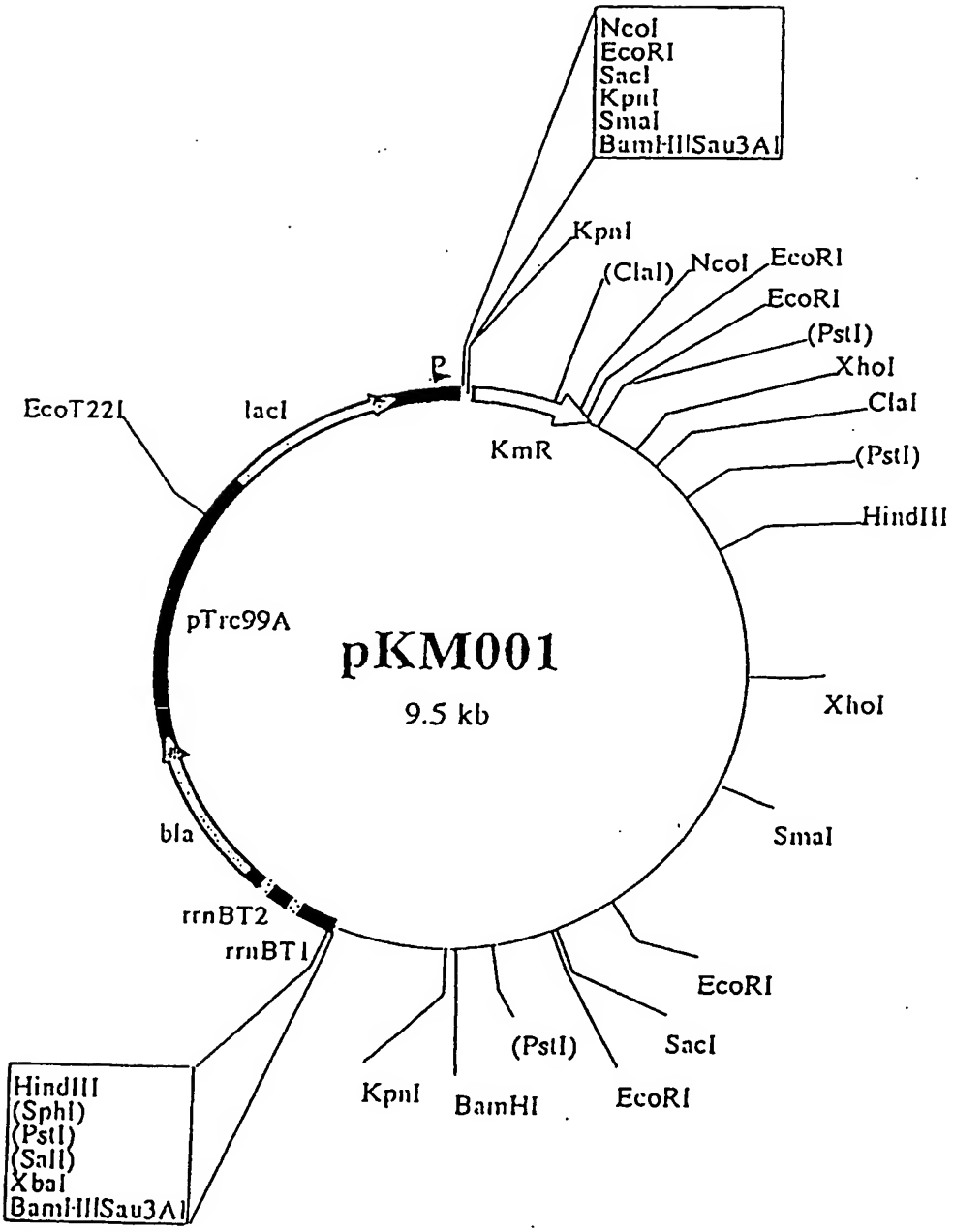
6. The plasmid vector according to claim 5, wherein the DNA region capable of replicating in a microorganism of the genus Rhodococcus is derived from a plasmid selected from pRC001, pRC002, pRC003 or pRC004.

7. A host cell transformed with the plasmid of claim 5 or 6.

8. The host cell of claim 7, which is a cell of a microorganism of the genus Rhodococcus or Escherichia coli.

9. Use of the gene of any one of claims 1 to 4 as a marker for the construction of a self-cloning system.

FIG. 1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SacI
KpnI
SmaI
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KpnI
(ClaI)
NcoI
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XhoI
ClaI
(PstI)
HindIII
XhoI
SmaI
EcoRI
SacI
(PstI)
EcoRI
KpnI
BamHI
HindIII
(SphI)
(PstI)
(SalI)
XbaI
BamHI/Sau3AI
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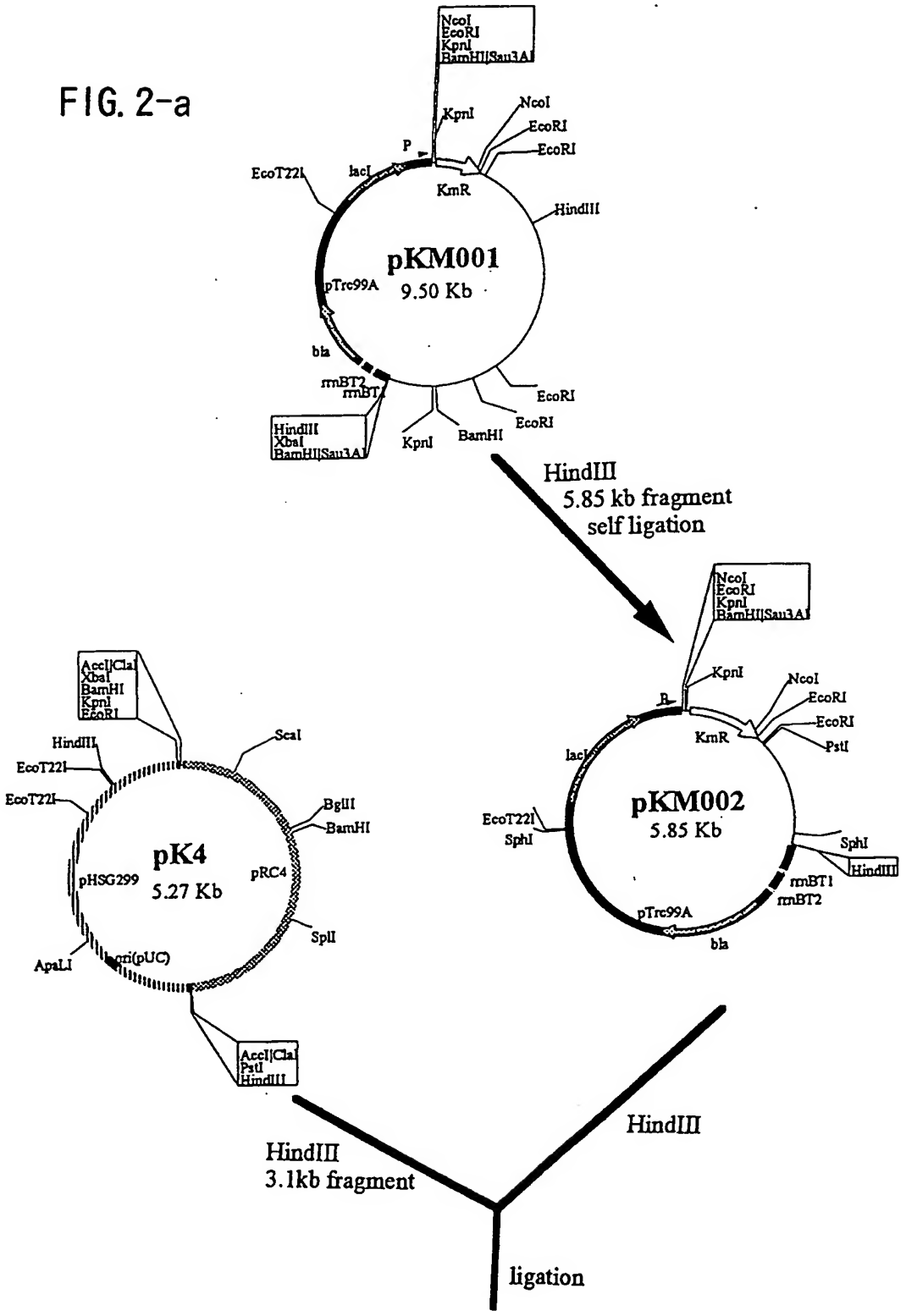
rrnBT2
bla
pTrc99A
EcoT22I
lacI
P
KmR
pKM001
9.5 kb

FIG. 2-a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KpnI
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EcoRI
EcoRI
P
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lacI
KmR
HindIII
pKM001
9.50 Kb
pTrc99A
bla
rrnBT2
rrnBT1
HindIII
XbaI
BamHI|Sau3AI
KpnI
BamHI
EcoRI
EcoRI
HindIII
5.85 kb fragment
self ligation
NcoI
EcoRI
KpnI
BamHI|Sau3AI
KpnI
NcoI
EcoRI
EcoRI
PstI
P
lacI
KmR
EcoT22I
SphI
pKM002
5.85 Kb
SphI
HindIII
rrnBT1
rrnBT2
pTrc99A
bla
AccI|ClaI
XbaI
BamHI
KpnI
EcoRI
HindIII
EcoT22I
EcoT22I
ScaI
BglII
BamHI
pK4
5.27 Kb
pHSG299
pRC4
SplI
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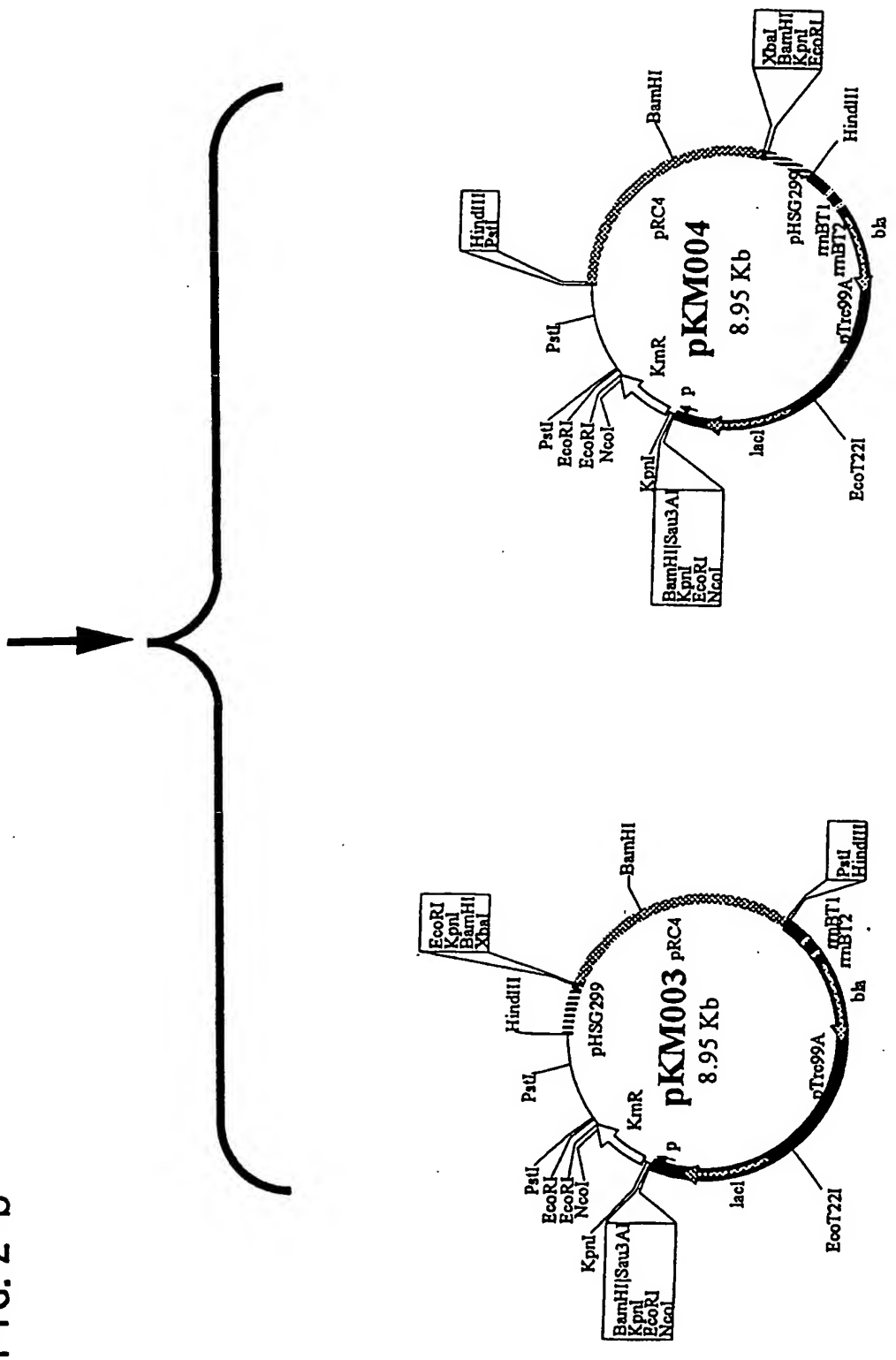
ori(pUC)
AccI|ClaI
PstI
HindIII
HindIII
3.1kb fragment
HindIII
ligation

FIG. 2-b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pHSG299
KmR
p
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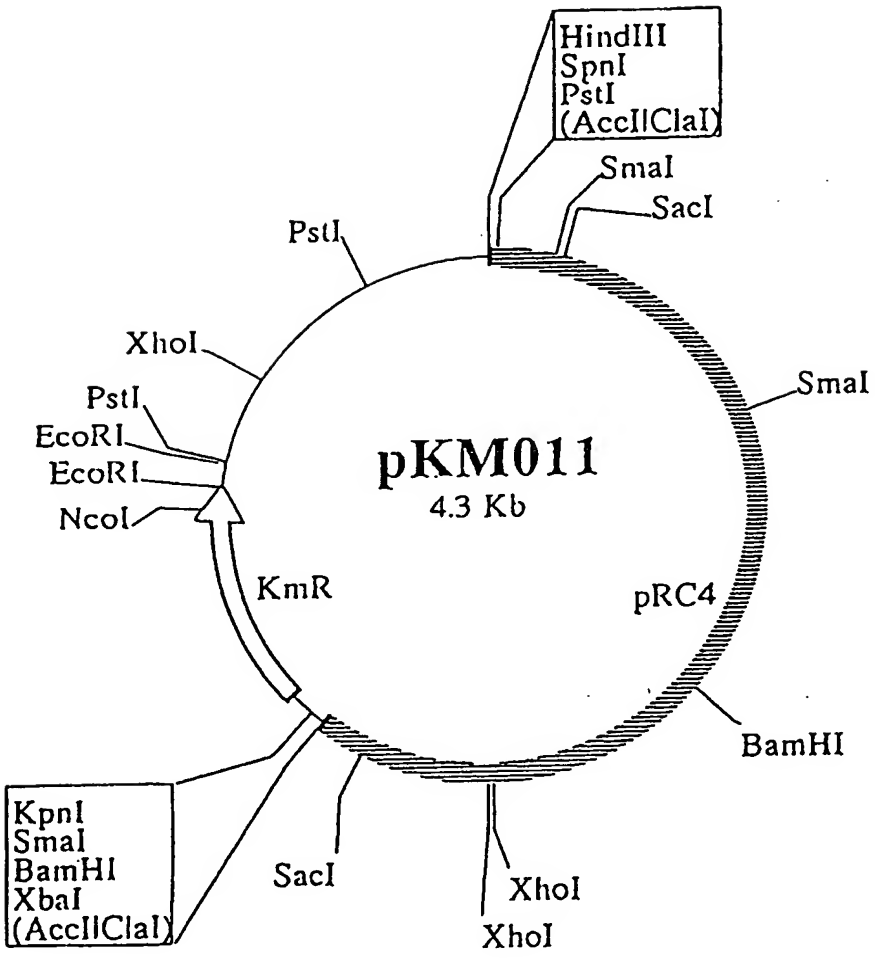
rmBT1
rmBT2
bla
EcoT22I
BamHI
HindIII
PstI
EcoRI
NcoI
KpnI
XbaI
Sau3AI
pKM003
8.95 Kb

FIG.3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SpnI
PstI
(AccIIClaI)
SmaI
SacI
PstI
XhoI
PstI
EcoRI
EcoRI
NcoI
KmR
pKM011
4.3 Kb
SmaI
pRC4
BamHI
KpnI
SmaI
BamHI
XbaI
(AccIIClaI)
SacI
XhoI
XhoI